Comparative immunogenicity of Foot and Mouth Disease Virus antigens in FMD-haemorrhagic septicaemia combined vaccine and FMD vaccine alone in buffalo calves

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Humoral immune response was evaluated by monitoring the serum antibody titres and virus specific IgM titres against Foot and Mouth Disease (FMD) virus antigens in serum samples obtained from different groups of calves inoculated with combined vaccine or FMD vaccine alone, on 0, 7, 14, 21, 28, 42 and 56 days post-vaccination (DPV). The cellular immune response was monitored by MTT based lymphoproliferation in peripheral blood mononuclear cell cultures. Higher liquid phase blocking (LPB) ELISA antibody titres were observed in calves receiving combined vaccine as compared to calves immunized with FMD vaccine alone with the peak titres in both the groups obtained on 21 days post-vaccination. However, the virus specific IgM titres were significantly higher in group of calves inoculated with combined vaccine than FMD vaccine alone. The lymphoproliferative responses against FMDV types O, A22 and Asia 1 in the groups receiving combined vaccine and FMD vaccine alone started increasing gradually after day 14 and reached peak levels on 28 DPV followed by a gradual decline subsequently. The group receiving combined vaccine showed higher proliferative responses on *in vitro* stimulation with FMD virus type O, whereas, with FMD virus type Asia 1, the responses were significantly higher on 14 and 21 DPV as compared to the group immunized with FMD vaccine alone. However, in the group receiving combined vaccine, the responses on *in vitro* stimulation with FMD virus type A22 were significantly higher than FMD vaccine alone group on all DPV except on 42 DPV.

**Keywords**: Buffalo calves, FMD+HS combined vaccine, Foot and Mouth Disease, Haemorrhagic septicaemia

Simultaneous occurrence of foot-and-mouth disease (FMD) and haemorrhagic septicaemia (HS) has been recorded for last 3-4 years, which are economically devastating diseases in our country. This was manifested in Moga district of Punjab in 1998 where large numbers of animals died. It is believed that FMDV produces a transient immunosuppression that provides an opportunity to predispose the animals to *Pasteurella multocida* infection resulting into the death of the animal. This pattern of combined outbreaks of FMD and HS has also been recorded in increasing number in Haryana during 2001 (ref. 2). This demands tremendous efforts to prevent the livestock from scourge of dual infection. Since both FMD and HS are endemic with widespread infection, the control programme to be considered should aim at systematic immunoprophylactic regimens, which can reduce the incidence as well as intensity of these diseases in endemic areas. One of the biggest constraints in obtaining adequate coverage is the poor response on the part of animal owners to vaccination campaigns. The response to HS vaccination campaign is usually better as farmer recognizes it as a fatal disease. Since FMD rarely kills the adult animal and animal usually recovers after a short period of illness, the response of farmers to FMD vaccination is less enthusiastic.

Recently, the vaccine manufacturers have come out with a combined FMD+HS oil adjuvant vaccine conferring immunity for longer duration to combat the menace of both of these infections. The administration of this vaccine, however, would not only reduce the cost and number of times a farmer needs to bring the animal for vaccination, but would also increase the number of animals vaccinated against FMD. But questions are raised whether in combined vaccines there is inter antigenic competition impairing the protective response to each of the constituent antigens. This demand necessitated a closer look at the effects, as measured by immune responses, of combined vaccination against these two diseases using commercially available vaccines. Very little is known about comparative immunogenicity of FMDV antigens in combined FMD+HS vaccine. In
the present study, the comparative immunogenicity of FMD+HS vaccine or oil adjuvant FMD vaccine alone was analyzed in buffalo calves.

Materials and Methods

**Virus**—FMD virus (FMDV) reference serotypes O, A22, C and Asia 1 (procured from Central Laboratory of Project Directorate on FMD; IVRI, Mukteshwar-Kumaon, Uttarakhal, India) were cultivated in Baby Hamster Kidney (BHK-21) clones 13-cell line using minimum essential medium (MEM, GIBCO BRL) supplemented with lactalbumin hydrolysate (LAH), tryptose soya broth, sodium bicarbonate, antibiotics and antifungal agents. The growth and maintenance media were supplemented with 10 and 2% fetal calf serum (FCS), respectively.

**Experimental animals**—A total of 15 buffalo calves, 3-4 month old, seronegative for FMD, maintained at Buffalo Research Center, College of Animal Sciences, CCS Haryana Agricultural University, Hisar, Haryana, were randomly selected. The animals were divided into 3 experimental groups containing 5 calves each. The first group served as control, second group was immunized with oil adjuvant FMD + HS combined vaccine and third group with oil adjuvant FMD vaccine alone. The animals were kept together under same managemental conditions. They were fed on green fodder and wheat *bhusa* (ad libitum), and concentrate mixture @ 0.75 kg/animal during whole period of study. Animals were dewormed 14 days before immunization.

**Vaccines**—Two types of vaccines were used: (a) The combined FMD+HS vaccine (Raksha Biovac, Indian Immunologicals) containing O, A22, C and Asia 1 and inactivated *Pasteurella multocida* culture emulsified with special mineral oil; and (b) Oil adjuvant polyvalent FMD vaccine (Raksha Ovac, Indian Immunologicals) containing O, A22, C and Asia 1 serotypes of FMDV, binary ethyleneimine inactivated and emulsified with special mineral oil.

**Collection and processing of samples**—Peripheral blood was collected aseptically by jugular venepuncture in test tubes containing heparin (10 IU/ml blood). Blood samples without heparin were also collected for serological studies. Serum was separated from the blood by placing tubes in slanting position for 1 hr at room temperature followed by 3-4 hr of incubation at 4°C. The tubes were then centrifuged at 1000 rpm for 15 min. The clear supernatant was collected as serum. For peripheral blood mononuclear cells (PBMC) heparinized peripheral blood samples collected on different days post-vaccination (DPV) were diluted with equal volume of RPMI-1640 (Sigma). Diluted peripheral blood samples (3 ml) were thus overlaid on layer of equal volume of Histopaque (1.077 ± 0.001 g/ml, Sigma). The tubes were centrifuged at 1500 rpm for 30 min at 4°C. The interface layer was harvested with Pasteur pipette and the cells were transferred to a sterilized test tube. The cells were then resuspended in RPMI-1640 medium and washed twice in the same medium. The viability of mononuclear cells was estimated by the trypan blue dye exclusion assay. The cells were suspended in RPMI-1640 at a final concentration of 1 × 10⁷ cells/ml.

**Humoral immune response**

**Liquid phase blocking ELISA**—The liquid phase blocking (LPB) ELISA was performed as described earlier using optimally diluted rabbit sera specific for all four FMDV serotypes as coating antibodies and anti-FMDV guinea pig serum as tracing antibodies.

**Estimation of titres**—The per cent inhibition in each well was calculated in relation to antigen control using the formula:

\[ \text{Inhibition} \% = \frac{\text{OD of test well} - \text{background OD}}{\text{OD antigen control} - \text{background OD}} \times 100 \]

The reciprocal of log₁₀ dilution corresponding to 50% inhibition was considered to be the titer of the serum.

**Indirect double antibody sandwich ELISA**—The test was done as described earlier. The ELISA plates were precoated with serotype specific rabbit anti-FMDV antibody overnight at 4°C. Plates were washed with phosphate buffer saline containing 0.01%Tween-20 (PBST), FMDV antigens at an optimum dilution added and incubated at 37°C for 1 hr. Plates were then washed thrice with PBST. Thereafter, double fold dilution of each sample (in duplicate) in diluent buffer (LAH 3% w/v, Newborn calf serum 5% v/v, Normal rabbit serum 5% v/v in PBST) was added and incubated at 37°C for 1 hr, washed with PBST followed by addition of biotinylated monoclonal antibody specific for bovine IgM (Sigma). After incubation at 37°C for one hr, Avidin-Biotin (1:2000, Sigma) complex was added and plates were further incubated for 30 min. Plates were then washed thrice and test was developed.

**Cell mediated immune response**

**Lymphoproliferative assay**—The test was performed using 3[4,5-Dimethylthiazol-2-yl]-2, 5-diphenyltetra-
zolium bromide; Thiazolyl-blue (MTT, Sigma) dye as per the method described earlier with slight modifications. Antigens were prepared by the same procedure as described earlier except that they were inactivated with binary ethylenimine (BEI). 2-bromoethylamine (BEA) (0.1 M) was maintained in 0.2 N NaOH for 1 hr at 37°C in water bath to obtain BEI, which is formed through cyclisation of BEA under alkaline conditions. To 9 ml of FMDV, 1 ml of 0.2 BEI was added and incubated at 37°C for 20 hr. The reaction was stopped by adding sodium thiosulphate at a final concentration of 2% and residual infectivity was examined by virus titration before use. Mock antigen was also prepared from cultures by the mean antibody titres against all the four FMDV types in both the vaccinated groups of calves were attained on 21 DPV followed by a gradual decline subsequently (Table 1). In the group of calves receiving FMD + HS combined vaccine, the antibody titres were significantly higher (P < 0.05) compared to calves receiving FMD vaccine alone against FMDV type O on 56 DPV, against FMDV type A22 on 21, 42 and 56 DPV and against FMDV type C on 7, 28, 42, and 56 DPV. However, the mean antibody titres against FMDV type Asia 1 in the group receiving FMD + HS combined vaccine were always higher than the group vaccinated with FMD vaccine alone with no significant variation on different DPV. The antibody titres in the control group were always less than 0.9 log10, the lowest dilution used in the assay.

Results

Humoral immune response — The peak antibody titres against all the four FMDV types in both the vaccinated groups of calves were attained on 21 DPV followed by a gradual decline subsequently (Table 1). In the group of calves receiving FMD + HS combined vaccine, the antibody titres were significantly higher (P < 0.05) compared to calves receiving FMD vaccine alone against FMDV type O on 56 DPV, against FMDV type A22 on 21, 42 and 56 DPV and against FMDV type C on 7, 28, 42, and 56 DPV. However, the mean antibody titres against FMDV type Asia 1 in the group receiving FMD + HS combined vaccine were always higher than the group vaccinated with FMD vaccine alone with no significant variation on different DPV. The antibody titres in the control group were always less than 0.9 log10, the lowest dilution used in the assay.

Virus specific IgM antibody response to FMDV — In the group of calves vaccinated with FMD+HS combined vaccine, the IgM antibody titres against FMDV type O and Asia 1 attained peak on 7 DPV and against FMDV type A22 on 14 DPV, which
declined subsequently (Table 2). However, the antibody titres in this group were significantly higher \((P<0.05)\) than the group vaccinated with FMD vaccine alone, except on 21 DPV against FMDV type O and on 28 DPV against FMDV type \(A_{22}\). The antibody titres in FMD+HS combined group against FMDV type Asia 1 were always higher \((P<0.05)\) than group receiving FMD vaccine alone. The antibody titres in the control group were always less than \(0.9 \log_{10}\), the lowest dilution used in the assay.

**Cellular immune response** — The lymphoproliferative responses of Con A-stimulated PBMC cultures (Fig. 1a), in the group receiving FMD+HS combined vaccine showed no significant variation compared to group receiving FMD vaccine alone except on day 14. The peak response in this group was observed on 28 DPV following which there was a decline on 42 and 56 DPV. The Stimulation Index (SI) values in the group receiving FMD vaccine alone showed gradual increase on 14 and 21 DPV, peaked on 28 DPV and declined thereafter. The proliferative responses in the control group were significantly lower.

Comparison of the SI values of FMDV type O stimulated PBMC cultures between groups receiving FMD+HS combined vaccine and FMD vaccine alone (Fig. 1b), revealed no significant differences. The proliferative responses in both the groups reached peak on 28 DPV and then declined gradually on 42 and 56 DPV.

The proliferative responses of the PBMC cultures stimulated with FMDV type \(A_{22}\) (Fig. 1c), of group vaccinated with FMD+HS vaccine were significantly higher \((P<0.05)\) as compared to the FMD vaccine alone group on all DPV except that response was of lower magnitude on 42 DPV. The proliferative

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**Table 2** — Serum IgM titres \((\log_{10})\) in buffalo calves vaccinated with FMD+HS combined or FMD vaccine alone against FMD virus type O, \(A_{22}\) and Asia 1

![Graph](image_url)

**Fig. 1** — Lymphoproliferative response of in vitro (a) Con A-stimulated PBMC cultures, (b) FMD virus type O-stimulated PBMC cultures, (c) FMD virus type \(A_{22}\)-stimulated PBMC cultures and (d) FMD virus type Asia 1-stimulated PBMC cultures obtained from buffalo calves vaccinated with FMD+HS combined or FMD vaccine alone

<table>
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<tr>
<th>Days post-vaccination</th>
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<th>FMD vaccine alone</th>
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<tr>
<td></td>
<td>O</td>
<td>(A_{22})</td>
<td>Asia 1</td>
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<td>7</td>
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<tr>
<td>14</td>
<td>&lt;0.9</td>
<td>&lt;0.9</td>
<td>(2.1 \pm 0.00^a)</td>
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<tr>
<td>21</td>
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<td>&lt;0.9</td>
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<tr>
<td>42</td>
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Mean with the same letters are not significantly different \((P<0.05)\).

N.D = Not done
responses in both the groups reached peak on 28 DPV and the declined gradually on 42 and 56 DPV.

The overall profile of lymphoproliferative responses produced by FMDV type Asia 1 stimulated PBMC from individual experimental groups at different DPV is shown in Fig. 1d. In group of calves receiving FMD+HS combined vaccine, the responses were significantly higher \((P<0.05)\) than the calves receiving FMD vaccine alone on 14 and 21 DPV while it was almost similar in PBMC culture obtained on 7, 28, 56 DPV. The proliferative responses in the control group were significantly lower.

**Discussion**

The comparative immunogenecity of FMDV antigens in FMD+HS combined and FMD vaccine alone has not been understood well. In the present study, comparative immunogenecity of FMDV antigens in FMD+HS combined and FMD vaccine alone was analyzed on the basis of humoral and cellular immune responses. Humoural response was analyzed by determining liquid phase blocking ELISA antibody titres and virus specific IgM antibody titres as measured by indirect double antibody sandwich ELISA. The cellular immune response was monitored using lymphoproliferative response to mitogen Con A and inactivated FMDV antigens (O, A22 and Asia 1).

The analysis of humoural immune response revealed comparatively higher antibody titres in the group of calves vaccinated with FMD + HS combined vaccine as compared to the calves vaccinated with FMD vaccine alone, though the differences were significantly higher on certain DPVs. Our results support the findings of Azfal and Muneech who reported higher antibody titres to FMDV antigens in rabbits using FMD+HS combined vaccine as compared to FMD alone vaccine. Enhanced humoral immune response against FMDV antigens has also been demonstrated by several other workers when FMD vaccine was simultaneously used with rabies\(^2\), HS\(^9\), anthrax\(^{14}\), and rinderpest\(^{17}\).

The earlier reports have compared immunogenecity utilizing virus neutralization (VN) and complement fixation (CF) tests\(^{19}\). However, LPB ELISA used in the present study correlates well to protective antibody levels\(^{11}\) and therefore, provides more substantial and conclusive data on comparative immunogenecity compared to those reported earlier.

The biological activities of antibodies are dependent on their isotype. The immunoglobulin class or subclass induced by an antigen influence the protective mechanisms involved in clearance of a particular antigen\(^{12}\). The present investigation, therefore, aimed to study virus specific (IgM) antibody response at different DPV. It is worthwhile to mention that virus specific IgM responses were significantly higher in group of calves vaccinated with FMD+HS combined vaccine than FMD vaccine alone. This may be attributed to the presence of bacterial antigens such as LPS in combined vaccine contributing towards recruitment of more B cells for effective presentation of FMDV antigens in combined vaccine.

In calves immunized with FMD vaccine alone, IgM antibody titres were highest against FMDV type Asia 1 (1.9 log\(_{10}\)) reaching peak on 21 DPV followed by type O (1.8 log\(_{10}\)) and minimum against type A22 (1.7 log\(_{10}\)). In earlier reports peak IgM responses were detected on 14 DPV, which declined around 28 DPV\(^{17}\). Further, it has been reported that LPS initiates proliferation and differentiation of B-lymphocytes, leading to the synthesis and secretion of non-specific IgM polyclonal antibodies\(^6\).

Generally, immune response against FMDV is evaluated on the basis of humoral immune response i.e. antibody titres. However, it has also been suggested that cellular immune responses too are required for providing protection in animals against FMD\(^{15,18}\). There seems to be no report on cellular immune response against FMDV antigens in combined vaccines. In the present study, the group receiving FMD+HS combined vaccine showed higher proliferative responses on *in vitro* stimulation with FMDV type O, whereas, with FMDV type Asia 1, the responses were significantly higher only on 14 and 21 DPV as compared to the group of calves receiving FMD vaccine alone. However, the proliferative responses on *in vitro* stimulation with FMDV type A22 in calves receiving FMD+HS combined vaccine were significantly higher as compared to the calves receiving FMD vaccine alone on all days post-vaccination except on 42 DPV.

The findings reported herein revealed that lymphoproliferative responses against FMDV antigens in combined vaccine were not hampered by the presence of *P. multocida* antigen. Moreover, the responses were higher on some of the DPV. The exact basis for potentiation of proliferative responses against FMDV antigens in combined vaccine is not well understood. However, there are reports which suggest that capsular antigen of *P. multocida* augment the lymphoproliferative responses in peripheral blood.
lymphocytes of cattle. Further studies are required to elucidate the possible immune mechanism involved in the potentiation of cellular immune responses to FMDV antigens in combined vaccine.

The findings of the present study clearly demonstrated that buffaloes may be safely vaccinated with FMD+HS combined vaccine without impairing the immune response against constituent FMDV antigens. Vaccination of large number of animals against important endemic diseases in country like India involves tremendous manpower and labour cost. Hence, there is a desire to develop alternative vaccination strategies in order to simplify the vaccination schedule both by decreasing the number of doses required for a vaccine course and increasing number of components per vaccine. In this scenario the approach of combined vaccine is a more prudent approach, as it would save labour cost as well as the cost of adjuvant.

References